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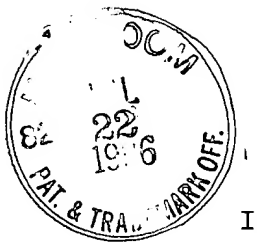

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8/23/96

PATENT DOCKET 175C2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	
Arjun Singh	Examiner: S. Priebe
Serial No. 08/448,946	Group Art Unit: 1805
Filed: 24 MAY 1995	
For: USE OF ALPHA FACTOR SEQUENCES IN YEAST EXPRESSION SYSTEMS	

DECLARATION REGARDING AMENDATORY MATERIAL
UNDER MPEP §608.01(p)(B)

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

I, Janet E. Hasak, do declare and say as follows:

1. I am the attorney of record in the above-identified application.

2. The material from U.S. Ser. No. 06/438,128 that is being inserted by the accompanying amendment into pages 8, 21, and 32 and the insertion of Figures 13, 14, 15A, 15B, and 16 into the above-identified application consist of the same material incorporated by reference in the above-identified referencing application, with the exceptions that obvious typographical errors have been corrected (such as "and" instead of "an" and "$MgCl_2$" instead of "MgCl2") and text on other subclones than that referenced and a Table I have been omitted as irrelevant. Further, the reference numbers are renumbered from references 50-54, 8, 9, 12, and 55-68 to references 63-84 respectfully and consecutively to conform with the numbering of references after reference 62 of the instant application, and the figure numbers are renumbered from Figures 1, 2, 3A, 3B, and 4 to Figures 13, 14, 15A, 15B, and 16, respectively, to conform with the numbering

of figures after Figure 12 of the instant application.

3. The material from U.S. Ser. No. 06/452,227 that is being inserted by the accompanying amendment into pages 8, 22, and 32 and the insertion of Figure 17 into the above-identified application consist of the same material incorporated by reference in the above-identified referencing application, with the exceptions that obvious typographical errors have been corrected (such as "ml" instead of "mls" and "more than" instead of "more") and the figure number is renumbered from Figure 1A to Figure 17, to conform with the numbering of figures after Figure 12 of the instant application and Figures 13-16 from the other referenced patent application.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: July 16, 1996

Janet E. Hasak
Janet E. Hasak
Reg. No. 28,616

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

CERTIFICATE BY "EXPRESS MAIL"
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I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.
KENNETH M. GOLDMAN
(Typed or Printed Name of Person Mailing Paper or Fee)
Kenneth M Goldman
(Signature of Person Mailing Paper or Fee)

BRAKE	:	
v.	:	Interference No. 102,728
SINGH	:	Examiner-in-Chief: R. Smith

DECLARATION OF ANTHONY J. BRAKE

Box Interference
Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

I, Anthony J. Brake, hereby declare:

1. I hold a Ph. D. in Biological Chemistry from the University of California, Los Angeles, and have over thirteen years of experience in molecular biology and recombinant DNA technology. I have authored or coauthored over 20 scientific publications. I am currently on a leave of absence from Chiron Corporation, which leave began in June 1991, when I was director of Yeast Biology. I began employment at Chiron on February 1, 1982. I am the inventor of the subject matter claimed in U.S. Patent No. 4,870,008. A copy of my *curriculum vitae* is submitted herewith as Exhibit 20.

2. I have read and understand the claims in the Singh application, U.S. Serial No. 552,719, and the Brak patent applications, U.S. Serial Nos. 457,325 ("Brake 1") and 522,909 ("Brake 2"). I have also read and understand the Count in this interference.

3. My review of the Brake 1 patent application indicates that it clearly discloses Saccharomyces α-factor constructs for secretory expression of heterologous genes, which constructs lack a dipeptidylaminopeptidase A ("DPAP A") site. This is disclosed, inter alia, in Brake 1 on page 3, line 30 to page 6, line 15.

4. My review of the Brake 1 patent application also indicates that it clearly discloses all embodiments of my invention known to me at the time of filing. Thus, the disclosure must have included the best mode contemplated by me for practicing my invention. A plasmid, pYEGF-8, disclosed in the application and containing the best DNA construct known to me and in my possession at the time of filing of Brake 1, was deposited at the ATCC on January 5, 1983.

5. As of January 1983 there were established and well known techniques available to persons of ordinary skill in the art by which constructs such as the one set forth in the Count could easily have been made:

a. For example, such constructs could have been made using site specific mutagenesis, a technique extensively used by January 1983 to modify DNA. This technique could have been

performed on (1) the construct ex mplified and deposited in the Brake 1 application, or (2) a similar construct made per the description in Brake 1. The technique of site specific mutagenesis (disclosed in Brake 2 and used to make constructs within the Count as shown at page 16, line 22 through page 18, line 16) was available in January 1983, and it would have been apparent to one of ordinary skill at that time to apply the technique to the material disclosed in Brake 1 to produce a construct of the Count.

b. An alternative technique would have been to digest the disclosed vector in Brake 1 with the restriction enzyme Hind III and then treat the digest with Bal 31. This technique would "chew" back the α-factor leader sequence to remove the Glu (or Asp)-Ala codons. One could easily have screened the Bal 31 digested material and isolated a fragment lacking the Glu (or Asp)-Ala codons that appear in the DPAP A site. Then the fragment would be blunt-end ligated to a foreign gene sequence using a suitable adaptor lacking the DPAP A site to form the spacerless construct of the count.

6. Once one constructed a construct lacking a DPAP A site, one of ordinary skill in the art would have recognized that it would be used in the identical way as the construct exemplified in the Experimental section starting on page 12 of Brake 1.

7. In 1983, I attended the 12th Annual UCLA Symposia, which were held between March 27 and April 30, 1983 in Keystone,

Colorado. On April 29, I gave a poster session and presentation in which I presented a series of *S. cerevisiae* α-factor constructs, including spacerless constructs, such as the ones described in Brake 2 and exemplified by the Count in the present interference.

8. During the April 29 poster session, I presented the results of my experiments demonstrating the successful construction of a yeast expression vehicle including the DNA construct embodied in claim 8 of the Singh application.

9. One construct in particular consisted of the *S. cerevisiae* α-factor leader sequence, terminating with the sequence encoding the first Lys-Arg dipeptide, connected in translation reading frame to the sequence encoding the first amino acid of mature epidermal growth factor (EGF). This DNA construct encoded a Lys-Arg C-terminal pre-pro-polypeptide of *S. cerevisiae* α-factor gene, and a DNA sequence encoding a mature protein heterologous to the yeast organism, wherein the sequences are joined directly together and do not include Glu (or Asp)-Ala repeats.

10. In addition to describing the above α-factor/EGF construct, I also presented results showing the use of that construct to transform a yeast organism, culture that organism, and recover mature EGF therefrom.

11. One skilled in the art, having attended my poster session and presentation on April 29, 1983, would have been able

to make and use the spacerless α-factor/EGF construct described above, or such constructs using a g ne other than that for EGF.

12. I have also read the pap r by Hitz man et al., Science (1983) 219:620-625, and understand its contents. Hitzeman et al. showed that it was possible to express human interferon in yeast. As one skilled in the art, I would have found it obvious to use the human interferon gene of Hitzeman et al. in the α-factor construct I disclosed at the Keystone Conference to obtain the invention of claims 20 and 21 of Singh. It would have been obvious to replace the human EGF gene disclosed by me with the human interferon gene of Hitzeman et al. to arrive at the invention of claims 20 and 21, since Hitzeman et al. clearly suggest the desirability of making interferon in yeast.

13. There are dozens of genera and tens of thousands of species of yeast. Yeast is a diverse group of microorganisms, most species of which are relatively poorly understood. Of these tens of thousands of species, only one species outside the genus *Saccharomyces*, namely *Kluyveromyces lactis*, is known to produce an α-factor related peptide. Nothing in the Singh application would adequately guide one skilled in the art to determine which yeast, other than the specifically disclosed *Saccharomyces* yeast, possess the disclosed characteristics and utility.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements

were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

3/1/92
Date

Anthony J. Brake

gldm040C

CERTIFICATE OF SERVICE

It is hereby certified that a copy of the foregoing DECLARATION OF ANTHONY J. BRAKE has been served by Express Mail upon the attorneys of record for the party Singh to this interference, on this 2nd day of March, 1992, at the following address:

R. Danny Huntington, Esq.
Burns, Doane, Swecker & Mathis
699 Prince Street, Suite 100
Alexandria, VA 22314

CERTIFICATE OF MAILING BY "EXPRESS MAIL"
"Express Mail" Mailing Label No. FB557260028 6US
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I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

KENNETH M. GOLDMAN
(Typed or Printed Name of Person Mailing Paper or Fee)

Kenneth M. Goldman
(Signature of Person Mailing Paper or Fee)

Thomas E. Ciotti

Thomas E. Ciotti
Reg. No. 21,013

Attorney for Brake

EB557266 [illegible]

KENNETH M. GOLDMAN

(Signature of Person Mailing Paper or Fee)

Atty Dkt 22300-20006.30

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

BRAKE	:	Interference No. 102,728
v.	:	
SINGH	:	Ronald H. Smith Examiner-in-Chief

BRAKE EXHIBITS ACCOMPANYING PRELIMINARY MOTIONS AND DECLARATIONS

Box Interference
Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Brake submits the following Exhibits 1 through 20 in connection with the Preliminary Motions and Declarations filed in the above-referenced Interference. These exhibits are referenced in the various motions and declarations and are submitted once in this form so as to simplify the record:

1. Brake U.S. Patent No. 4,870,008;

2. List of Attendees to 12th Annual UCLA Symposia held in Keystone, Colorado;

3. Hitzeman et al. Science: (1983) 219:620-625;

4. Papers Nos. 10 and 14 of Chang prosecution U.S. Serial No. 488,337 (matured as Chang et al., U.S. Patent No. 5,010,003);

5. Paper No. 16, pages 11-14, of Chang prosecution U.S. Serial No. 488,337 (matured as Chang et al., U.S. Patent No. 5,010,003);

6. Paper No. 35 of Chang prosecution U.S. Serial No. 488,337 (matured as Chang et al., U.S. Patent No. 5,010,003);

7. Curriculum Vitae of Dr. Patricia Tekamp-Olson;

8. Curriculum Vitae of Dr. Guy Mullenbach;

9. Page 12 of Brake 1, U.S. Serial No. 457,325;

10. H. Gregory and B.M. Preston, Int. J. Peptide Protein Res 9: 107-118 (1977);

11. Preliminary Amendment filed concurrently with Brake 3, U.S. Serial No. 081,302;

12. Paper No. 5 (Interference Request) of Singh U.S. Serial No. 552,719 (Singh 3);

13. Form PTO-436 from Brake 1 showing 1/12/83 filing date with Preliminary Amendment; Form PTO 436-from Brake 2 showing 8/12/83 filing date; 5/14/84 Telephone Restriction of Brake 1; and first Office Action, dated 6/27/84 of Brake 1;

14. Ex Parte Singh, Paper No. 29 of U.S. Serial No. 06/506,098 (Singh 2);

15. Paper No. 18 (Examiner Interview Summary) of Brake U.S. Serial No. 522,909;

16. Pap r No. 19 (Amendment) of Brake U.S. Serial No. 522,909;

17. Hitzeman U.S. Patent No. 4,775,622;

18. U.S. Serial No. 06/457,325 (Brake 1) -- served upon Attorneys of Record for Party Singh only;

19. U.S. Serial No. 06/522,909 (Brake 2) -- served upon Attorney of Record for Party Singh only; and

20. Curriculum Vitae of Dr. Anthony Brake.

Respectfully submitted,

By: Thomas E. Ciotti

Thomas E. Ciotti
Registration No. 21,013

COUNSEL FOR THE PARTY BRAKE

MORRISON & FOERSTER
545 Middlefield Road, Suite 200
Menlo Park, California 94025
Telephone: (415) 813-5600
Fax: (415) 327-2951

Of Counsel:
Robert P. Blackburn
Registration No. 30,477
Chief Patent Counsel
Chiron Corporation
4560 Horton Street
Emeryville, California 94608
(510) 655-8730

Journal of Cellular Biochemistry
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SUPPLEMENT 7B, 1983

12th Annual
UCLA SYMPOSIA

Abstracts

MARCH 27 – APRIL 30, 1983

Alan R. Liss, Inc., New York

[illegible]

Pre-registered Conferees

David [illegible]
Wistar Institute
Philadelphia PA 19104

James J. Anderson
Genex Corporation
Gaithersburg MD 20877

Yair Argon
MRC Center, Hills Rd
Cambridge CB2 2QH ENGLAND

Vytas Bankaitis
Univ N Carolina
Chapel Hill NC 27514

Sue Bartlett
Louisiana State Univ
Baton Rouge LA 70803

Philip Bassford
Univ North Carolina
Chapel Hill NC 27514

Linda Baum
Duke Univ Med Ctr
Durham NC 27710

Spencer Benson
NCI-FCRF
Frederick MD 21701

Rex Bitner
Biosciences
St Paul MN 55144

Grant Bittner
AMGen
Thousand Oaks CA 91320

Kenneth Blumenthal
Univ Cincinnati
Cincinnati OH 45267

Robert Blumenthal
NIH
Bethesda MD 20205

Jef D. Boeke
Mass Inst Technology
Cambridge MA 02139

Alan Boyd
Univ California,San Diego
La Jolla CA 92093

Anthony Brake
Chiron Corporation
Emeryville CA 94608

Jerry Brown
Univ Colorado Hlth Sci
Denver CO 82062

Bernard Brownstein
Abbott Laboratories
Chicago IL 60064

Wolfgang Bruns
GBS Mascheroder
D 3300 Braunschweig FRG

Hermann Bujard
Univ Heidelberg
Heidelberg FRG 6900

Ronald Cape
Cetus Corporation
Berkeley CA 94710

Marian Carlson
Columbia University
New York NY 10032

Gary Ceccarini
VA Medical Center
San Francisco CA 94142

Peter Chan
Dow Chemical Company
Midland MI 48640

Chung Nan Chang
Genentech, Inc
San Francisco CA 94080

[illegible] Chang
Cetus Corporation
Berkeley CA 947[illegible]

Andrew Charles
ICI PLC
Cheshire WA7 4QE ENGLAND

Jean-Pierre Chenevert
University of Quebec
Montreal H3C 3P8 CANADA

[illegible]
[illegible] California,San Diego
La Jolla CA [illegible]2093

Chin H. Chung
Harvard Medical School
Boston MA 02115

Kiomin Chung
Washington State Univ
Pullman WA 99164

Aaron [illegible]
Mass Inst Technology
Cambridge MA 02139

Patrick Connor
Univ California
Los Angeles CA 90024

Claude Cote
Jewis General Hospital
Montreal H3T 1E2 CANADA

Michael [illegible]
Univ Colorado
Boulder CO 80309

Norman Curthoys
Univ Pittsburgh Schl Med
Pittsburgh PA 15261

Valarie Darby
Univ Wisconsin
Madison WI 53706

Lance Davidow
Pfizer Central Res
Groton CT [illegible]

Bernard Davis
Harvard Med School
Boston MA 02115

Jacques Deshusses
University
Geneva 1211 SWITZERLAND

[illegible]
[illegible]
[illegible]

Nanni Din
Nordisk Gentofte
Gentofte DK-2820 [illegible]

Kurt Doege
Univ California
Los Angeles CA 90024

[illegible]
Univ Texas Hlth Sci Ctr
San Antonio TX 78284

[illegible]
Yale University
New Haven CT 06511

[illegible]
ICI Pharmaceuticals
Cheshire ENGLAND

John E[illegible]son
VA Wadsworth Med Ctr
Los Angeles CA 90073

Scott Emr
Univ California
Berkeley CA 94720

Donald Engelman
Yale University
New Haven CT 06511

Brad Erickson
Baylor Col Medicine
Houston TX 77030

Tina Etcheverry
Genentech Inc
San Francisco CA 94080

S. Fahnestock
Genex Corporation
Gaithersburg MD 20877

John Fessler
Univ California
Los Angeles CA 90024

Niels Fiil
Novo Research Inst
DK 2880 Bagsvaerd DENMARK

Mary Lynn Fink
NIH
Bethesda MD 20205

Yolanta Fishman
Tufts Med Schl
Boston MA 02111

Jan-Ingmar Flock
G.D. Searle Res & Develpt
High Wycombe ENGLAND

Robert Florkiewicz
The Salk Institute
San Diego CA 92138

C. Fred Fox
Univ California
Los Angeles CA 90024

Robert O. Fox
Yale University
New Haven CT 06510

Mark Frana
Uniformed Services Univ
Bethesda MD 20814

Victor Fried
St Jude Hospital
Memphis TN 38101

Susannah Gal
National Cancer Inst
Bethesda MD 20205

Mary-Jane Gething
Cold Spring Harbor Labs
Cold Sprg Hrbr NY 11724

[illegible] Ghosh
UMDNJ-Rutgers Med Schl
Piscataway NJ 08854

Reid Gilmore
Rockefeller University
New York NY 10021

Werner Goebel
Inst f Genetik und Mikrob
Wurzburg D-8700 FRG

David Goeddel
Genentech Inc
San Francisco CA 94080

Alfred Goldberg
Harvard Medical Schl
Boston MA 02115

Marian Gorecki
Biotechnology General Corp
Rehovot 76326 ISRAEL

[illegible]
Genentech Inc
San Francisco CA 94080

Michael [illegible]
St Louis Univ Schl Med
St Louis MO 63104

Reza [illegible]
Albert Einstein Col Med
Bronx NY 10461

[illegible] Greenberg
Harvard University
Cambridge MA 02138

Leslie [illegible]
Univ Amsterdam
Amsterdam NETHERLANDS

Georges Guellaen
Institut Pasteur
Paris 75015 FRANCE

Mark Guyer
Genex Corp
Gaithersburg MD 20877

David L. Hare
AMGen Development, Inc
Boulder CO 80301

James F. Hare
Oregon Hlth Sci Univ
Portland OR 97201

Richard L. Harkins
Genentech
San Francisco CA 94080

Ed Heath
Univ Iowa Coll Med
Iowa City IA 52242

Linda M. Hendershot
Univ Alabama
Birmingham AL 35294

M.J. Hofnung
Institut Pasteur
Paris 75015 FRANCE

Cornelis P. Hollenberg
Univ Dusseldorf
D-4000 Dusseldorf F.R.G.

David Hondred
Univ Wisconsin
Madison WI 53706

[illegible]
[illegible] Med Ctr
[illegible]

[illegible]
[illegible]
[illegible]

[illegible]
Univ [illegible]
Birmingham AL 35294

[illegible]
Fukuoka Univ Schl Med
Fukuoka 814-01 JAPAN

[illegible]
State Univ New York
Stony Brook NY 11794

[illegible]
Harvard [illegible]
Cambridge MA [illegible]

David Jackson
Genex Corp
Gaithersburg MD 20877

Maria J. [illegible]
T-NEMC
Boston MA [illegible]

George A. [illegible]
Univ Michigan
Ann Arbor MI [illegible]

James Kadonaga
Harvard Univ
Cambridge MA 02183

Chris Kaiser
Mass Inst Tech
Cambridge MA 02139

Arnold [illegible]
St. Louis Univ Med Schl
St. Louis MO 63104

H. Gobind Khorana
Mass Inst Tech
Cambridge MA 02139

Daniel L. Kilpatrick
Roche Inst Molec Biol
Nutley NJ 07110

Thomas M. Kloppel
VA Med Ctr
Denver CO 80220

Jeremy R. Knowles
Harvard Univ
Cambridge MA 02138

Tadahiko Kohno
Collaborative Res Inc
Lexington MA 02173

Lillian A. Koro
Duke Univ Med Ctr
Durham NC 27710

Richard A. Kramer
Hoffmann-La Roche Inc
Nutley NJ 07110

Carol Kumamoto
Harvard Med Schl
Boston MA 02115

J. Oliver Lampen
Rutgers Univ
Piscataway NJ 08854

Denis LeBel
Univ Sherbrooke
Sherbrooke CANADA

Hope Kiebke
Yale University
New Haven CT 06510

Mark Lively
Bowman Gray Schl Med
Winston-Salem NC 27103

Stephen Lory
Harvard Med Schl
Boston MA 02115

Michael [illegible]
Codon Labs
Brisbane CA 94005

Ardythe McCracken
Univ Colorado Hlth Sci Ctr
Denver CO 80262

Joan McEwen
Univ Colorado
Boulder CO 80309

Gary McKnight
Univ Washington
Seattle WA 98195

Nancy McQueen
UCLA Schl Medicine
Los Angeles CA 90024

Carolyn Machamer
Duke University
Durham NC 27710

Catherine Mackey
Pfizer Central Labs
Groton CT 06340

Vivian MacKay
Zymos Corporation
Seattle WA 98105

Pamela Maher
Univ Calif,San Diego
La Jolla CA 92093

Nancy [illegible]
Univ Texas Hlth Sci Ctr
Dallas TX [illegible]

Mark Matteucci
Genentech, Inc
San Francisco CA 94080

Maija Mednieks
NIH
Bethesda MD 20205

Susan Michaelis
Harvard Med Schl
Boston MA [illegible]

Jonathan Mielenz
CPC International
Argo IL 60501

Robert Mierendorf
Univ Wisconsin
Madison WI 53706

James [illegible]
Lilly Research Labs
Indianapolis IN 46285

Raymond Mosteller
Univ South Calif Schl Med
Los Angeles CA 90033

Debi Nayak
UCLA Schl Medicine
Los Angeles CA 90024

Penelope Nazos
University of Michigan
Ann Arbor MI 48109

Gregory Nelson
Calif Institute Technology
Pasadena CA 91109

Otto [illegible]
Univ South Dakota
Vermillion SD 57069

[illegible]
[illegible]
[illegible]

Thalia Nicas
Oregon Hlth Sci Univ
Portland OR 97201

[illegible]
Cetus Corporation
Berkeley CA 94710

[illegible]
Hynson Wescott & Dunning
Baltimore MD 21030

[illegible]
University of Florida
Gainesville FL 32610

David Ogrydziak
Univ California, Davis
Davis CA 95616

Yoshiko Ohno-Iwashita
Univ California
Los Angeles CA 90024

Kenneth Olden
Howard Univ Cancer Ctr
Washington D.C. 20060

Bauke Oudega
Vrije Universiteit
Amsterdam NETHERLANDS

Dale Oxender
University Michigan
Ann Arbor MI 48109

Ilkka Palva
Univ Helsinki
Helsinki 29, FINLAND

J. Brian Parent
Howard Univ Cancer Ctr
Washington D.C. 20060

Michael Parker
Zymos Corporation
Seattle WA 98103

Gregory Payne
Univ California, Berkeley
Berkeley CA 94720

Janice Pero
BioTechnica Internatl
Cambridge MA 02140

Vincent Pigiet
Johns Hopkins Univ
Baltimore MD 21218

Livia Poenaru
Inst Pathologie Molec
Paris FRANCE

Jeffrey Price
Cetus Corporation
Berkeley CA 94710

John Pringle
University of Michigan
Ann Arbor MI 48109

Alan Proctor
Pfizer Central Res
Groton CT 06340

Vito Quaranta
Scripps Clinic
La Jolla CA 92037

Steven Quay
Stanford Univ Schl Med
Stanford CA 94305

Richard Racusen
Univ Maryland
College Pk MD 20742

J. L. [illegible]
Pfizer Central Res
Groton CT 06415

Linda Randall
Washington State Univ
Pullman WA 99164-4630

Beth Rasmussen
Univ North Carolina
Chapel Hill NC 27514

[illegible]
[illegible] Research Labs
[illegible] Triangle Pk NC 27709

Martin Rechsteiner
University of Utah
Salt Lake City UT 84112

John Re
Genentech Inc
San Francisco CA 94080

[illegible]
[illegible] Wadsworth Med Ctr
Los Angeles CA 90073

John Riordan
Hosp for Sick Children
Toronto Ontario CANADA

Harry Rittenhouse
Abbott Laboratories
North Chicago Il 60044

[illegible]
Univ North Carolina
Chapel Hill NC 27514

Marta Sabara
Univ Saskatchewan
Saskatoon Saskt CANADA

David Sabatini
New York Univ Med Ctr
New York NY 10016

Joseph Sambrook
Cold Spring Harbor Lab
Cold Sprg Hrbr NY 11724

Padmini Sampathkumar
Harvard Med Schl
Boston MA 02115

Court Saunders
Monsanto Company
St Louis MO 63167

Jaymie Sawyer
Univ Wisconsin
Madison WI 53706

Randy Schekman
Univ Calif, Berkeley
Berkeley CA 94720

Neal Scherberg
Univ Chicago
Chicago IL 60637

David Schlossman
Stanford Univ Med Ctr
Stanford CA 94305

Albert Schmitz
Biogen S.A.
Geneva SWITZERLAND

Wolfgang Schneider
Univ Texas Hlth Sci Ctr
Dallas TX 75235

Ronald Schoner
Lilly Research Labs
Indianapolis IN 46285

Nancy Schwartz
Univ Chicago
Chicago IL 60643

Jere Segrest
Univ Alabama
Birmingham AL 35294

Albert Einstein Col Med
Bronx NY 10461

William Sly
Washington University
St Louis MO 63178

Ranga Srinivas
Univ Alabama
Birmingnam AL 35294

Michael Stephens
Harvard University
Cambridge MA 02138

Susan Straley
Univ Alabama
Birmingham AL 35294

Jim Swartz
Genentech Inc
San Francisco CA 94080

Ginan Tennekoon
Johns Hopkins Med Schl
Baltimore MD 21205

Masao Tokunaga
Uniformed Serv Univ
Bethesda MD 20814

Frederic Troy
Univ Calif Schl Med
Davis CA 95616

George Vlasuk
State Univ New York
Stony Brook NY 11794

Barbara Wallner-Philipp
Biogen
Cambridge MA 02142

Joel Weiner
Univ Alberta
Edmonton Alb CANADA

Michael Whittaker
Univ Colorado Hlth Sci
Denver CO 80262

Jo Wise
Univ Calif Schl Med
San Francisco CA 94143

Andrew Wright
Tufts Medical Schl
Boston MA 02111

McGill University
Montreal H3g 1Y6 CANADA

Darwin Smith
Rice University
Houston TX 77251

Stephen Stanl
Biogen S.A.
Geneva SWITZERLAND

Tom Stevens
Univ California, Berkeley
Berkeley CA 94720

Sid Suggs
AMGen Inc
Thousand Oaks CA 91320

Harry Taber
Albany Med College
Albany NY 12208

Jeremy Thorner
Univ Calif, Berkeley
Berkeley CA 94720

Paul Tolstoshev
Transgene S.A.
Strasbourg FRANCE

Eugene Tustanoff
Univ Western Ontario
London N6A 5C1 CANADA

Michael Vodkin
USAMRILD-Ft Detrick
Frederick MD 21701

Kenneth Walsh
Univ Washington
Seattle WA 98195

Sandra White
Howard Univ Med Schl
Washington D.C. 20060

William Wickner
Univ California
Los Angeles CA 90024

William Wold
St Louis Univ Scl Med
St Louis MO 63110

Henry Wu
Uniformed Services Univ
Bethesda MD 20814

NCI-FCRF
Frederick MD 21701

John Smith
Pennsylvania State Univ
University Pk PA 16802

Donald Steiner
Univ Chicago
Chicago IL 60637

Roselynn Stevenson
University of Guelph
Guelph Ontario CANADA

Joyce Sutcliffe
Abbott Laboratories
N Chicago IL 60064

Allen Taylor
Harvard University
Cambridge MA 02138

David Titus
Univ Wisconsin
Madison WI 53706

Jill Trewhalla
Yale University
New Haven CT 06511

Thierry Vernet
National Res Council
Ottawa K1A 0R6 CANADA

Charles Waechter
Univ Maryland Med Schl
Baltimore MD 21201

Michael Waterman
Univ Texas Hlth Sci Ctr
Dallas TX 75235

Stephen White
Univ Calif, Irvine
Irvine CA 92717

John Wills
Univ Alabama
Birmingham AL 35294

Paul Wolfe
Univ Calif, Los Angeles
Los Angeles CA 90024

Ryland Young
Texas A&M University
College Station TX 77843

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KENNETH M. GOLDMAN

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Kenneth M Goldman

(Signature of Person Mailing Paper or Fee)

Atty Dkt 22300-20006.30

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

BRAKE	:	Interference No. 102,728
v.	:	
SINGH	:	Ronald H. Smith Examiner-in-Chief

MOTION (4) BY THE PARTY BRAKE PURSUANT TO 37 C.F.R. § 1.633(a) FOR JUDGMENT ON THE GROUND THAT THE CLAIMS OF PARTY SINGH ARE UNPATENTABLE UNDER 35 U.S.C. §§ 102(a) AND 103

Box Interference
Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

I. STATEMENT OF RELIEF REQUESTED

The party Brake (hereinafter "Brake") hereby moves, pursuant to the provisions of 37 C.F.R. § 1.633(a), for judgment on the grounds that: (a) claims 8 and 19 of the application U.S. Serial No. 07/552,719 (hereinafter "Singh 3"), of party Singh ("Singh") are unpatentable under 35 U.S.C. § 102(a), based on the public knowledge of the invention of those claims on April 29, 1983, which, on the present record, is before the invention thereof by

Singh; and (b) claims 20 and 21 of the application of Singh are unpatentabl under 35 U.S.C. § 103, based on the aforementioned public knowledg , further in view of Hitz man et al., Science (1983) 219:620-625 (submitted herewith as Exhibit 3).

II. STATEMENT OF FACTS AND LAW IN SUPPORT OF MOTION

A. Claims 8 and 19.

35 U.S.C. § 102 provides in part that a person shall be entitled to a patent unless --

> a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent.

Anticipatory knowledge under § 102(a) means public knowledge of a complete and operative invention, Rosemount, Inc. v. Beckman Instruments, Inc., 218 U.S.P.Q. 881 (C.D. Cal. 1983), aff'd, 221 U.S.P.Q. 1 (Fed. Cir. 1984), including each and every element of the claimed invention. In re Bond, 15 U.S.P.Q.2d 1566, 1567 (Fed. Cir. 1990).

Claim 8 of the Singh application reads:

> A yeast expression vehicle comprising the DNA sequence encoding a lys arg C-terminal pre-pro peptide of yeast alpha factor gene operably connected in translation reading frame without intervening Glu (or Asp)-Ala dipeptide repeats to a DNA sequence encoding a mature protein heterologous to the yeast organism, wherein the DNA encoding all of the Glu (or Asp)-Ala dipeptide repeats has been deleted from the pre-pro peptide of the yeast alpha factor DNA.

Thus the elements of claim 8 are:

> A yeast expression vehicle which comprises:
>
> (a) a DNA sequence encoding a Lys-Arg C-terminal pre-pro peptide of yeast alpha factor gene;
> (b) a DNA sequence encoding a mature protein heterologous to yeast; wherein
>
> (c) the sequences are joined directly together and do not include Glu (or Asp)-Ala repeats.

These elements must be compared with the public knowledge on April 29, 1983. The Declaration of Dr. Anthony Brake (hereinafter "Dr. Brake Decl.", submitted herewith) demonstrates that the invention of claim 8 was disclosed to the public prior to the claimed filing date of claim 8, which is June 20, 1983. (See "Singh Miscellaneous Motion (1) Pursuant to 37 C.F.R. § 1.635" (to Deny Benefit) filed February 26, 1992.)

The 12th Annual UCLA Symposia were held between March 27 and April 30, 1983 in Keystone, Colorado. Dr. Brake Decl. ¶ 7. Many molecular biologists and yeast geneticists, including several Genentech researchers, were in attendance. See List of Attendees, attached hereto as Exhibit 2. On April 29, 1983, Dr. Brake gave a poster session and presentation disclosing a spacerless α-factor construct, such as that in the Count. Dr. Brake Decl. ¶ 7.

On that poster, Dr. Brake presented his results demonstrating the successful construction of a yeast expression vehicle including the DNA construct embodied in claim 8 of the

Singh application. A construct on Dr. Brake's poster consisted of the *S. c revisiae* α-factor leader sequ nc , terminating with the sequence encoding the first Lys-Arg dipeptid , connected in translation reading frame to the sequence encoding the first amino acid of mature epidermal growth factor (EGF). Dr. Brake Decl. ¶ 9.

Thus, the yeast expression vehicle disclosed by Dr. Brake at the Keystone Conference included a DNA construct encoding: (1) a Lys-Arg C-terminal pre-pro peptide of yeast α-factor gene; and (2) a DNA sequence encoding a mature protein heterologous to the yeast organism; wherein (3) the sequences are joined directly together and do not include Glu (or Asp)-Ala repeats. Dr. Brake Decl. ¶ 9. One skilled in the art at that time would have been able to make and use this DNA construct. Id. ¶ 11. As such, Dr. Brake's presentation constituted public knowledge of an invention containing each and every element of claim 8 recited above, and is thus a complete anticipation under 35 U.S.C. § 102 of the invention of that claim. See American Standard, Inc. v. Pfizer, Inc., 14 U.S.P.Q.2d 1673, 1709 & n.42 (D. Del. 1989) (contents of a speech given at a scientific conference in the U.S. constitutes prior art under the public knowledge provision of 35 U.S.C. § 102(a)).

Claim 19 of Singh 3 reads:

> 19. A process for obtaining a mature protein heterologous to yeast as a product of yeast expression, which process comprises:

(a) transforming a yeast organism with an expression vehicle comprising the DNA sequ nce encoding a lys arg C-terminal pre-pro peptide of yeast alpha factor operably connected in translation reading frame without intervening Glu (or Asp)-Ala dipeptide repeats to a DNA sequence encoding a mature protein heterologous to the yeast organism, wherein the DNA encoding all of the Glu (or Asp)-Ala dipeptide repeats has been deleted from the pre-pro peptide of the yeast alpha factor DNA;

(b) culturing the transformed organism; and

(c) recovering mature protein from the culture having an N-terminal amino acid sequence identical to that of the protein from natural sources.

Claim 19 relates to a process for making a mature protein heterologous to yeast involving transforming a yeast organism with the DNA construct of claim 8, culturing the organism, and then recovering the mature protein.

At the Keystone Conference, Dr. Brake, in addition to disclosing the α-factor/EGF construct described above, also disclosed the use of that construct to transform a yeast organism, culture that organism, and recover mature EGF therefrom. Dr. Brake Decl. ¶ 10. Therefore, Dr. Brake's disclosure also anticipated the invention of claim 19.

Thus, claims 8 and 19 read on unpatentable subject matter as defined by 35 U.S.C. § 102(a).[1/]

1/ Dr. Brake's public disclosure does not raise a patentability issue as to the claims in the Brake patent. 35 U.S.C. § 102(a) only bars a patent where the invention was "known or used by

B. Claims 20 and 21.

35 U.S.C. § 103 states, in part:

> A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.

An invention may be obvious over a combination of prior art if there is a teaching or suggestion in the art that would lead one of ordinary skill in the art to make the combination. Smithkline Diagnostics, Inc. v. Helena Laboratories Corp., 8 U.S.P.Q.2d 1468, 1475 (Fed. Cir. 1988).

Claims 20 and 21 in Singh 3 depend upon claims 8 and 19, respectively, and thus contain the elements of those claims, and are further limited to human α-interferon. Dr. Brake's disclosure of April 29, 1983, discussed in detail in Section A, supra, teaches every element of Singh's claims 20 and 21, but does not explicitly teach the specific expression of human α-interferon. However, Dr. Brake's disclosure would have enabled one skilled in the art to make and use the spacerless α-factor construct using a gene other than that for EGF. Dr. Brake Decl. ¶ 11.

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others," not by the inventor himself.

Hitzeman et al., Science (1983) 219:620-625 (Exhibit 3), describes the expression and secretion of human interferon in yeast, and has a publication date of February 11, 1983.[2/] Thus, Hitzeman et al., in combination with Dr. Brake's disclosure, teach all the elements of claims 20 and 21. It would have been obvious to one skilled in the art to use the human interferon gene of Hitzeman et al. in the α-factor system of Brake to obtain the invention of claims 20 and 21. Dr. Brake Decl. ¶ 12. Hitzeman et al. showed that it was desirable to express human interferon in yeast. It would therefore have been obvious to replace the human EGF gene of the Brake disclosure with the human interferon gene of Hitzeman et al. to arrive at the invention of claims 20 and 21. Id. ¶ 12.

Claims 20 and 21 are thus unpatentable under 35 U.S.C. § 103 over Dr. Brake's disclosure in view of Hitzeman et al.

CONCLUSION

For the foregoing reasons, Brake respectfully submits that claims 8 and 19 in party Singh's application are unpatentable under 35 U.S.C. § 102(a), and claims 20 and 21 in party Singh's application are unpatentable under 35 U.S.C. § 103. Brake notes for the record that these grounds of unpatentability do not apply

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2/ In the alternative, Brake also relies on U.S. Patent No. 4,775,622 to Hitzeman et al. (submitted herewith as Exhibit 17), which discloses the same expression and secretion of human interferon in yeast, and has a reference date of November 1, 1982 pursuant to 35 U.S.C. § 102(e).

to Brake because they are based on Brak 's own disclosure which is not prior art to Brake.

Respectfully submitted,

Thomas E. Ciotti
Registration No. 21,013

COUNSEL FOR THE PARTY BRAKE

MORRISON & FOERSTER
545 Middlefield Road, Suite 200
Menlo Park, California 94025-3471
Telephone: (415) 813-5600
FAX: (415) 327-2951

Of Counsel:

Robert P. Blackburn
Registration No. 30,447
Chief Patent Counsel
Chiron Corporation
4560 Horton Street
Emeryville, California 94608
(510) 655-8730

G0405

ABSTRACT

HUMAN BLOOD PLASMA CONTAINS TWO SIMILAR POLYPEPTIDES, INSULIN-LIKE GROWTH FACTORS I AND II (IGF I, 70 AMINO AICDS, AND IGF II, 67 AMINO ACIDS), WHICH COMPRISE A GROUP OF INSULIN-LIKE GROWTH HORMONE-DEPENDENT PEPTIDES WHICH ARE BELIEVED TO MEDIATE THE GROWTH PROMOTING ACTIONS OF GROWTH HORMONE. THE NUCLEOTIDE SEQUENCES CODING FOR THESE POLY-PEPTIDES HAVE BEEN SYNTHESIZED ON SOLID SUPPORT BY A MODIFICATION OF PHOSPHORAMIDITE COUPLING PROCEDURES. SINGLE STRAND SEQUENCES AVERAGING 25 BASES IN LENGTH WERE SYNTHESIZED AND PURIFIED. DUE TO PARTICULARLY LARGE OVERLAPS BETWEEN STRANDS, ASSEMBLY OF EACH OF THESE GENES FROM THEIR OLIGOMERS WAS ACHIEVED IN A SINGLE ANNEALING AND LIGATION EXPERIMENT WITHOUT THE PIECEMEAL ASSEMBLY APPROACH CONVENTIONALLY REPORTED. CODONS CHOSEN FOR THESE SYNTHESES FOLLOWED THE FOLLOWING PRINCIPLES: (1) CODONS USING THE YEAST CODON BIAS WERE SELECTED TO MAXIMIZE EXPRESSION IN THIS ORGANISM. (2) RESTRICTION SITES WERE BUILT INTO THE GENES AT CONVENIENT LOCATIONS IN ORDER TO ALLOW FOR CONSTRUCTION OF 6 DIFFERENT IGF I/IGF II GENE HYBRIDS AND POLYPEPTIDE HYBRID MOLECULES. YEAST CELLS TRANSFORMED WITH PLASMIDS CONTAINING THESE GENES PRODUCED BIOLOGICALLY ACTIVE IGF I AND IGF II.

INTRODUCTION

IT IS SUSPECTED THAT SOMATIC GROWTH WHICH FOLLOWS THE ADMINISTRATION OF GROWTH HORMONE IN VIVO IS MEDIATED THROUGH A FAMILY OF MITOGENIC, INSULIN-LIKE PEPTIDES WHOSE SERUM CONCENTRATIONS ARE GROWTH HORMONE DEPENDENT. AMONG THESE PEPTIDES INSULIN-LIKE GROWTH FACTORS I AND II HAVE BEEN ISOLATED IN LIMITED AMOUNTS FROM HUMAN SERUM AND SEQUENCED.[1,2]

IT HAS BEEN OF PARTICULAR SCIENTIFIC AND CLINICAL INTEREST TO US TO PRODUCE RELATIVELY LARGE QUANTITIES OF THESE GROWTH FACTORS. TO THIS END WE PRESENT HERE (1) CHEMICAL TECHNIQUES FOR THE SYNTHESIS OF GENES CODING FOR THESE GROWTH FACTORS AND (2) RECOMBINANT DNA TECHNIQUES WHICH UTILIZE A YEAST/a-FACTOR EXPRESSION SYSTEM THAT ACHIEVES SECRETION OF THESE PROTEINS FROM YEAST.

RINDERKNECKT & HUMBEL, J. BIOL. CHEM., (1978).
RINDERKNECKT & HUMBEL, FEBS LETTERS, (1978).

METHODS

DESIGN

THE CODON SEQUENCES OF SYNTHETIC GENES CODING FOR IGF-I AND II WERE ESTABLISHED BY UTILIZING THEIR PUBLISHED PROTEIN SEQUENCES[1,2] (FIGURE 1).

CODONS WERE SELECTED SUCH THAT:

(1) EXPRESSION IN YEAST MIGHT BE MAXIMIZED BY UTILIZING THOSE CODONS MOST FREQUENTLY FOUND IN THE GLYCOLYTIC ENZYMES OF YEAST (i.e. BY MAINTAINING THE YEAST CODON BIAS).
(2) ASSEMBLIES WERE FACILITATED BY REMOVAL OF LONG HOMOLOGOUS STRETCHES WHICH MIGHT CAUSE INCORRECT ANNEALING OF OLIGOMERS.
(3) CONVENIENT RESTRICTION SITES WERE GENERATED SO THAT VARIOUS HYBRID IGF-I/IGF-II GENE AND POLYPEPTIDE CONSTRUCTIONS CAN BE SYNTHESIZED.
(4) UNDESIRABLE RESTRICTION SITES WERE REMOVED.

OLIGOMERIC COMPONENTS WERE SYNTHESIZED SO AS TO YIELD MAXIMUM OVERLAPS AND TO MAKE MOST EFFICIENT USE OF SEGMENTS HOMOLOGOUS TO BOTH GENES (FIGURE 3).

DNA SYNTHESIS

OLIGOMERS AVERAGING 25 BASES IN LENGTH (FIGURE 2) WERE SYNTHESIZED ON A SOLID SUPPORT BY A PHOSPHORAMIDITE COUPLING APPROACH.

ASSEMBLIES

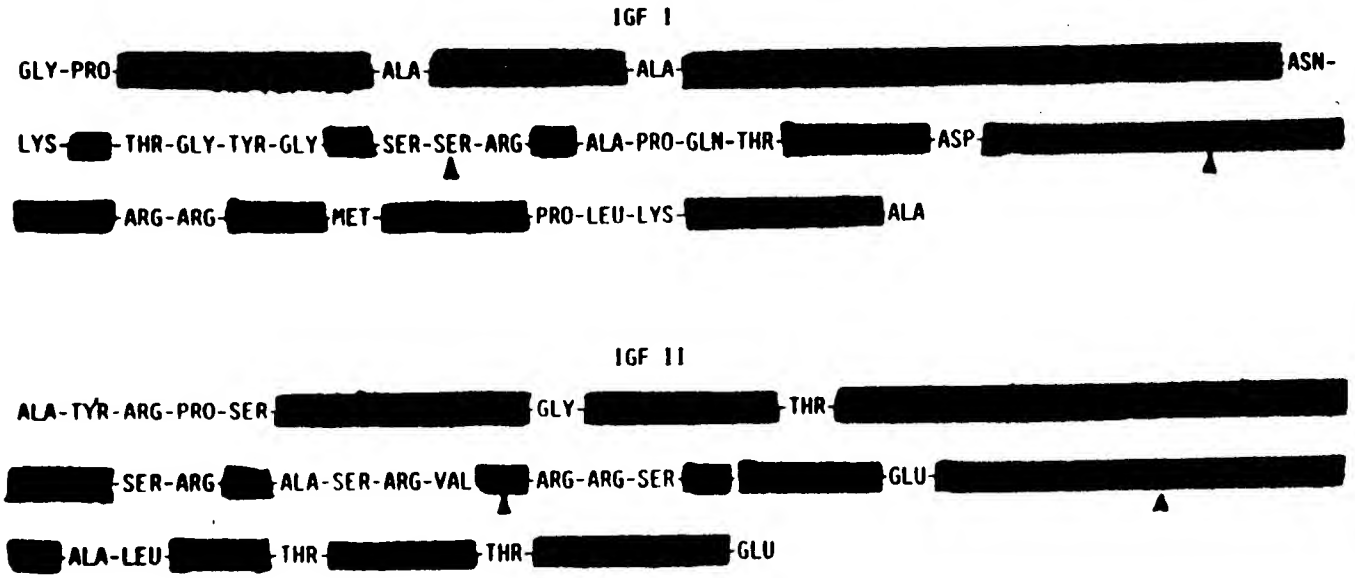
THE ENZYMATIC LIGATION OF EACH GENE WAS ACHIEVED IN A SINGLE ANNEALING/LIGATION POOL RATHER THAN BY WAY OF PIECEMEAL ASSEMBLIES USUALLY REPORTED (FIGURE 3). CLONED CONSTRUCTIONS WERE SEQUENCED BY THE MAXAM GILBERT PROCEDURE (FIGURE 4).

EXPRESSION

PROTEIN CODING REGIONS WERE DIRECTLY FUSED TO THE YEAST α-FACTOR LEADER CODING REGION IN SUCH A WAY THAT THE REGIONS CODING FOR THE α-FACTOR PROCESSING SITES ARE MAINTAINED (FIGURE 5). YEAST CELLS TRANSFORMED WITH SUCH PLASMID CONSTRUCTS APPEAR TO SECRETE IGF-I OR IGF-II. PRELIMINARY RESULTS FROM RADIOIMMUNOASSAYS (BY MARTIN SPENCER, CHILDREN'S HOSPITAL, S.F.), RECEPTOR BINDING ASSAYS (M. SPENCER), A BIOASSY (PIGEON CROP GROWTH ACTIVITY), AND MOLECULAR WEIGHT DATA (FIGURE 6) SUPPORT THE IDENTITIES OF THESE HORMONES.

FIGURE 1.

FIGURE 1. PROTEIN SEQUENCES OF IGF I[1] AND II[2]

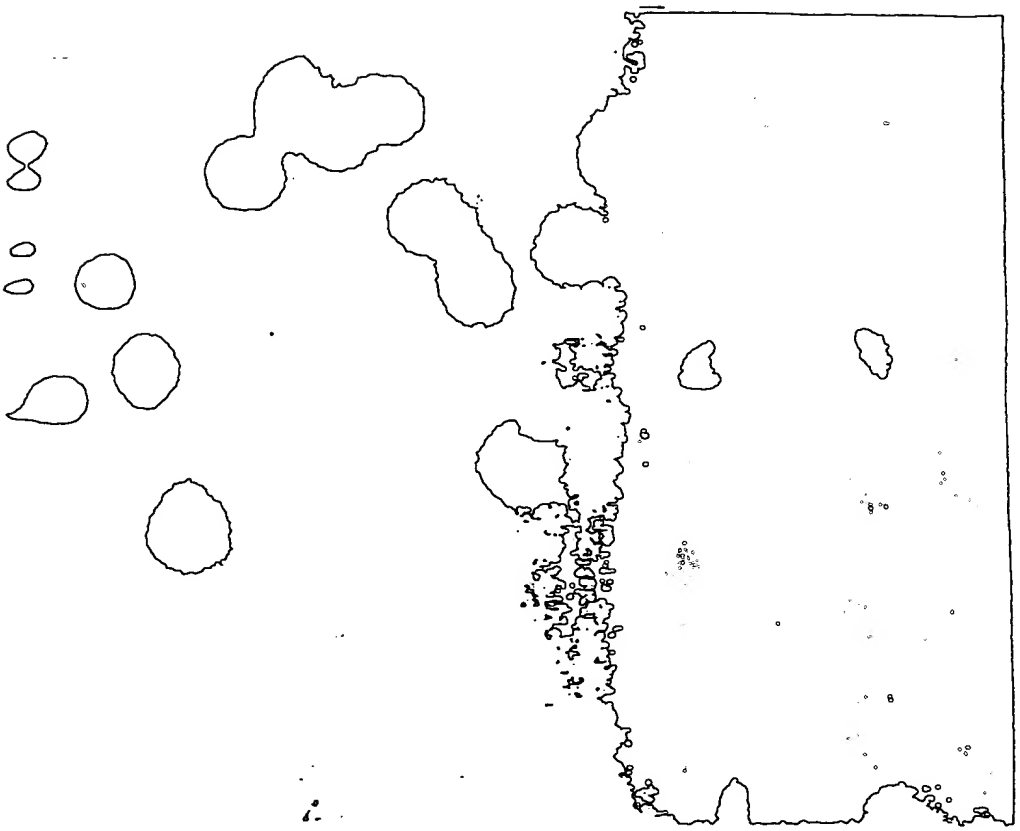


HOMOLOGOUS PORTIONS SHOWN IN YELLOW.

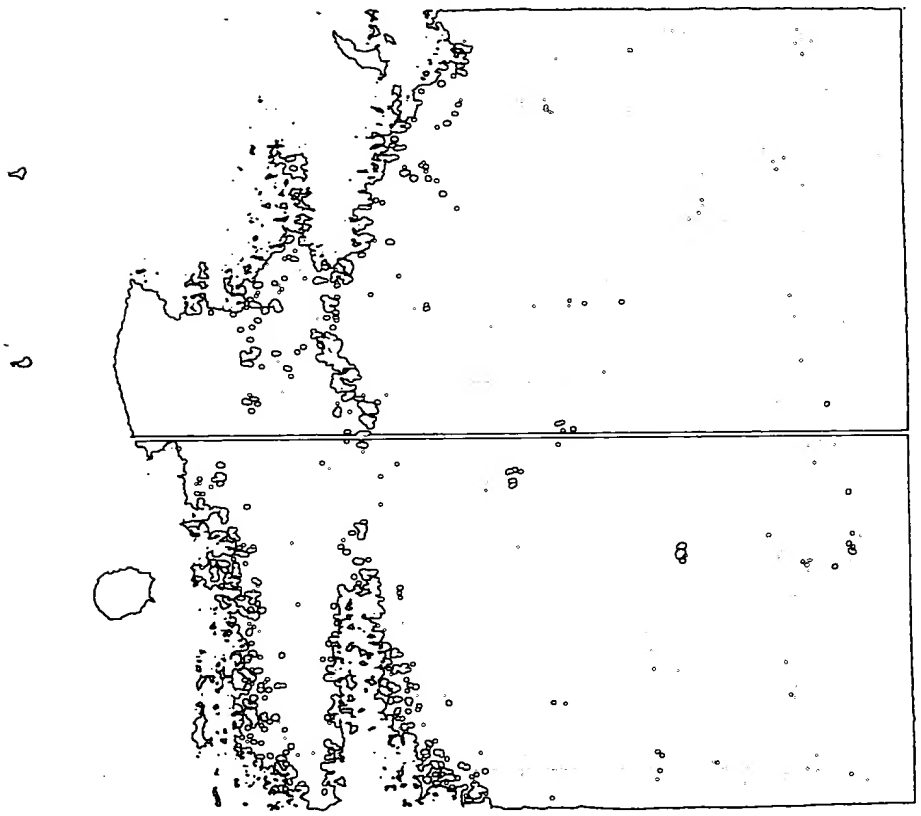
1. RINDERKNECHT & HUMBEL, J. BIOL. CHEM., (1978).

2. RINDERKNECHT & HUMBEL, FEBS LETTERS, (1978).

IGF I



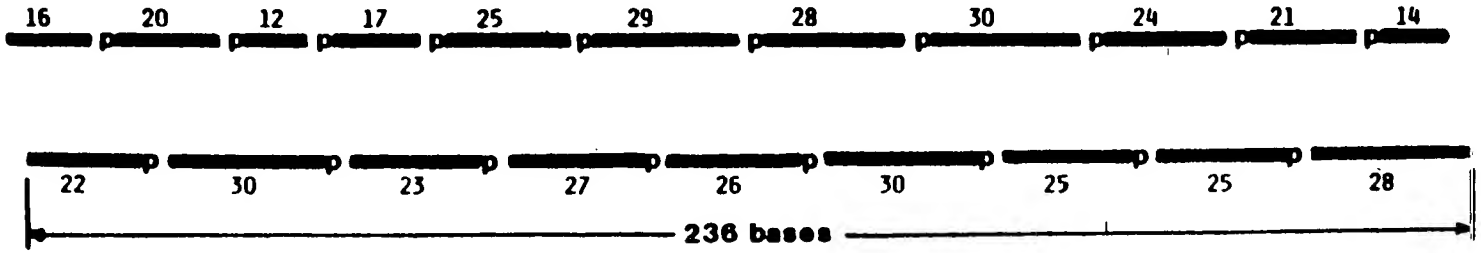
IGF II



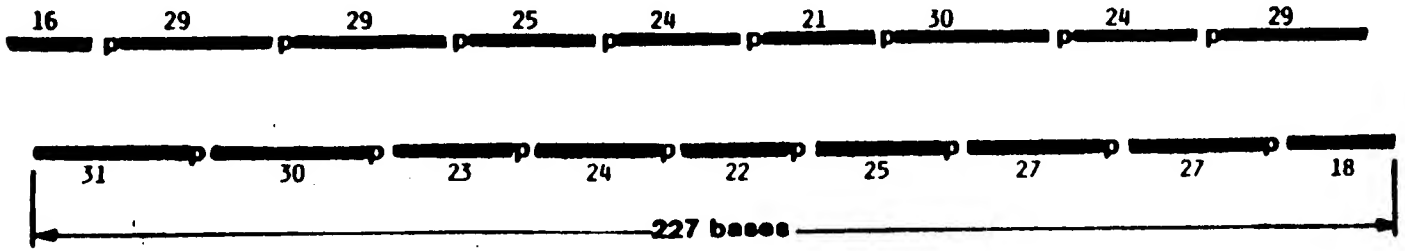
THESE OLIGONUCLEOTIDES AVERAGING 25 BASES IN LENGTH WERE SYNTHESIZED ON A SOLID SUPPORT BY A PHOSPHORAMIDITE COUPLING PROCEDURE (URDEA, MERRYWEATHER, MULLENBACH, COIT, HEDERLEIN, VALENZUELA & BARR, PNAS, SUBMITTED FOR PUBLICATION) AND SIZED BY POLYACRYLAMIDE GEL ELECTROPHORESIS.

FIGURE 3. ASSEMBLY SCHEME

IGF-I



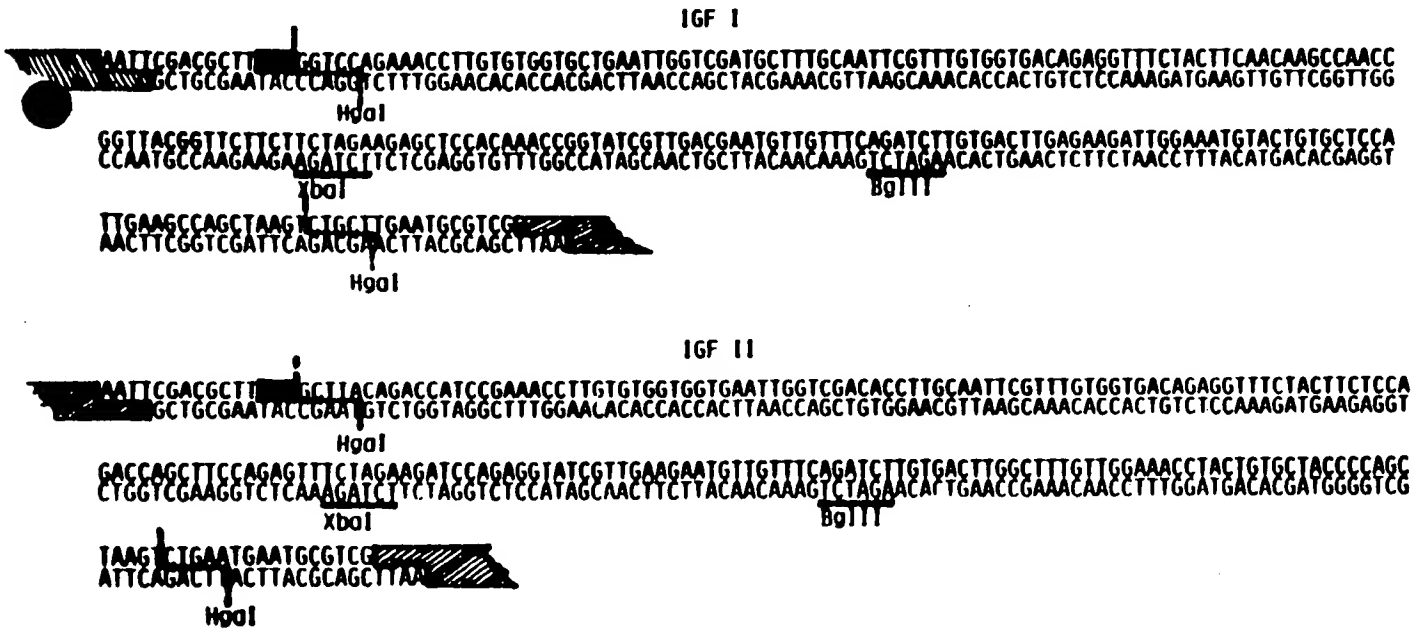
IGF-II



LARGE OVERLAPS BETWEEN STRANDS OF ABOUT 25 BASES LONG PERMIT ASSEMBLY IN A SINGLE ANNEALING AND LIGATION POOL RATHER THAN PIECEMEAL ASSEMBLIES PREVIOUSLY REPORTED.

FIGURE 4.

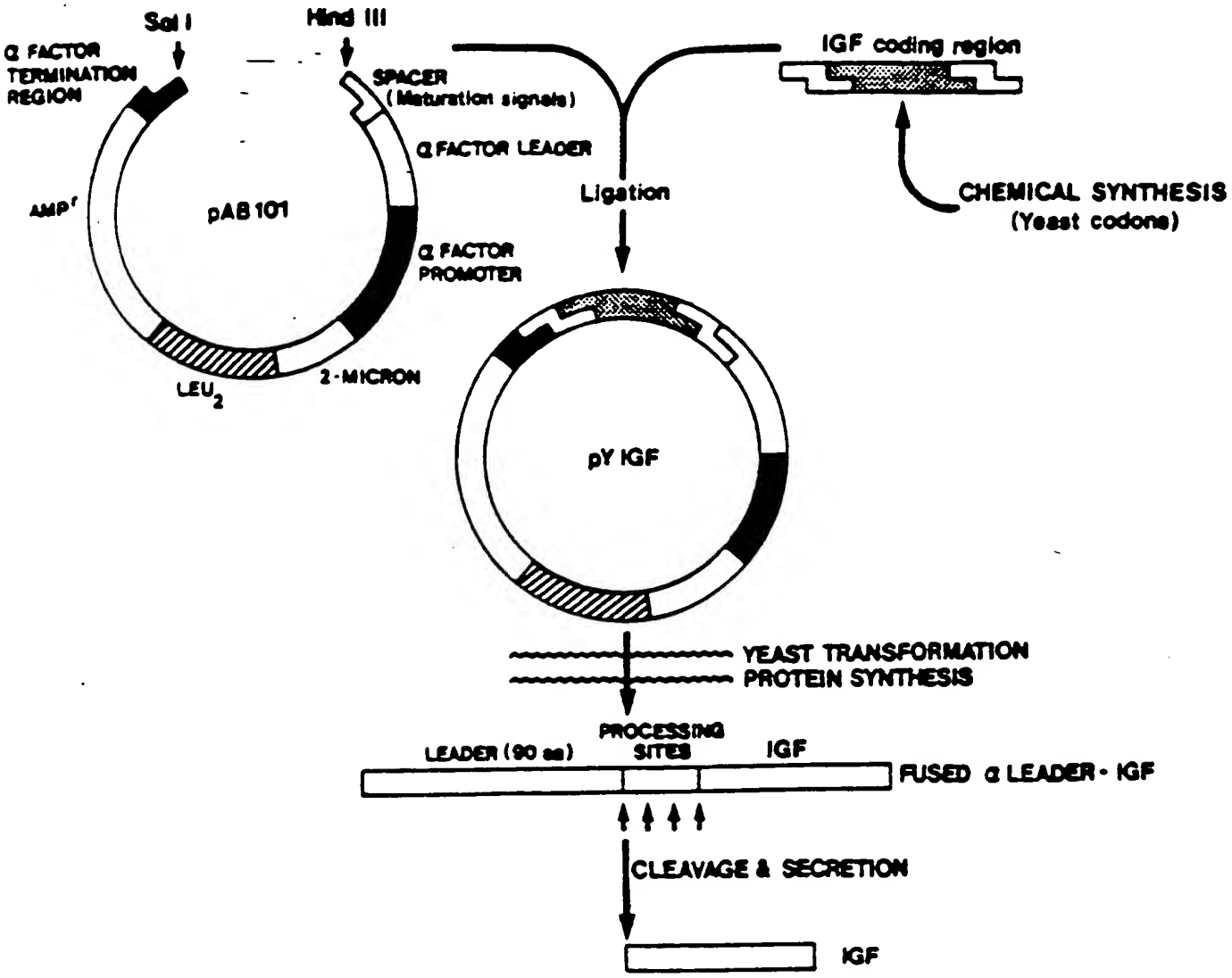
FIGURE 4. DNA SEQUENCING RESULTS



THE SYNTHETIC DOUBLE-STRANDED CONSTRUCTIONS WERE CLONED INTO EcoRI DIGESTED pBR328 AND THE INSERT SEQUENCED BY THE MAXAM AND GILBERT PROCEDURE.

RESTRICTION SITES IN RED HAVE BEEN BUILT INTO THE GENE FOR POTENTIAL HYBRID IGF I/IGF II CONSTRUCTIONS.

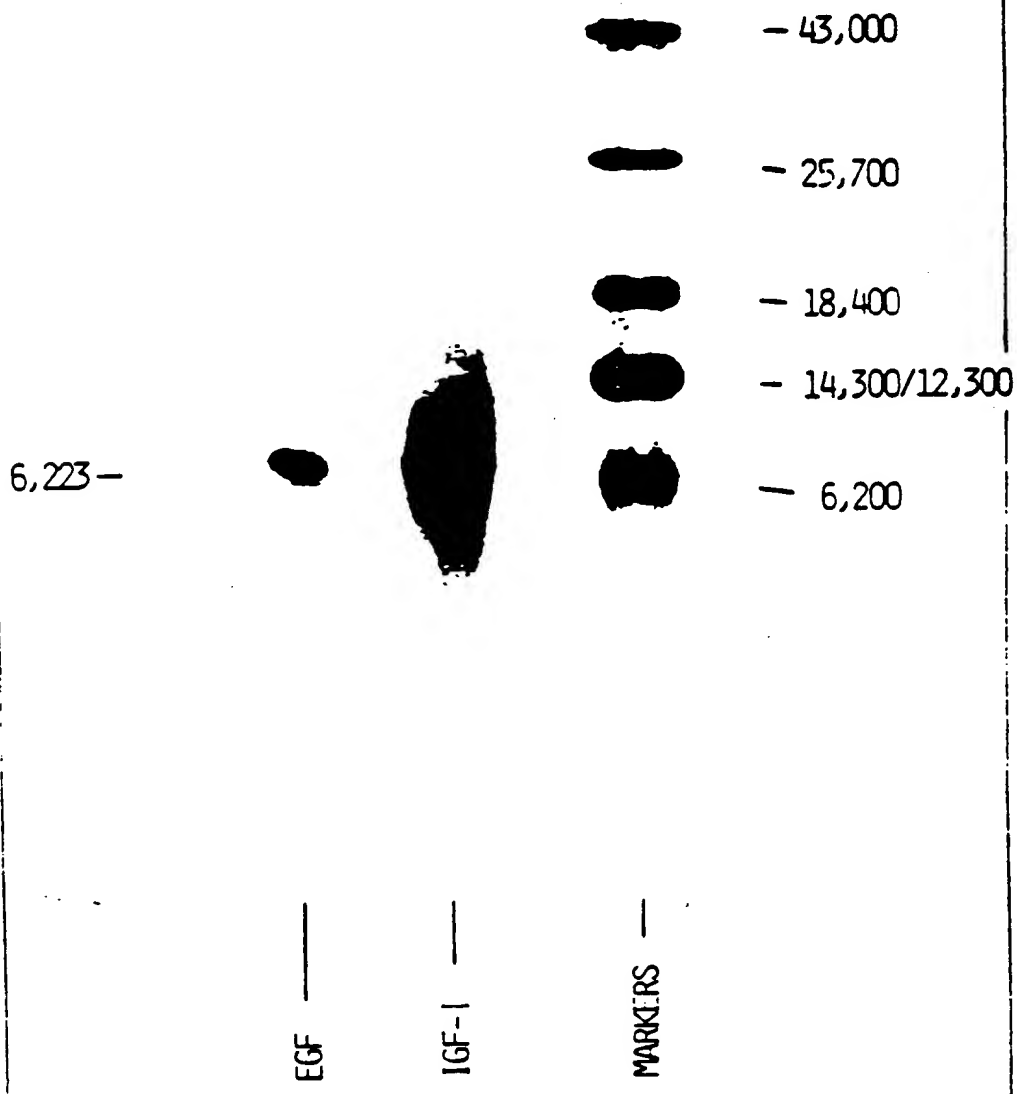
FIGURE 5. PROCESSING AND SECRETION OF PROTEINS IN YEAST



FIRST THE CODING SEQUENCE TO BE CLONED INTO YEAST WAS PRECISELY EXCISED FROM THE pBR328/IGF PLASMID WITH HgaI (WHICH CUTS OUTSIDE ITS RECOGNITION SITE). THIS FRAGMENT WAS THEN EQUIPPED WITH LINKERS SUCH THAT THE REGIONS CODING FOR THE α-FACTOR PROCESSING SITES ARE MAINTAINED, AND THEN CLONED INTO THE YEAST α-FACTOR VECTOR. APPROPRIATE POSTTRANSLATIONAL PROCESSING IS ACHIEVED UPON SECRETION OF IGFs FROM YEAST.

FIGURE 6.

FIGURE 6. SDS POLYARCRLAMIDE GEL ELECTROPHORESIS



ONLY THE YEAST CULTURE SUPERNATANT WAS SUBMITTED TO BIOREX-70 CATION EXCHANGE CHROMATOGRAPHY PRIOR TO SDS GEL ELECTRO-PHORESIS.

CONCLUSIONS

GENES CODING FOR HUMAN INSULIN-LIKE GROWTH FACTORS I AND II HAVE BEEN SYNTHESIZED BY CHEMICAL MEANS. EACH WAS ABLE TO BE ASSEMBLED IN A SINGLE POOL FROM THEIR OLIGOMERIC COMPONENTS. IGF-I AND IGF-II ARE EXPRESSED AND SECRETED IN YEAST AT LEVELS AS HIGH AS ABOUT 1 MG/LITER OF CELL CULTURE (IGF-I). THUS, FOR THE FIRST TIME, SUFFICIENT QUANTITIES OF THESE PROTEINS ARE AVAILABLE FOR EXTENSIVE STUDY.